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Darier disease: molecular study of a two-generation Portuguese family

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Abstract

Background Darier Disease is a rare autosomal dominant disorder, which predominantly affects the skin of individuals regardless their gender or ethnicity. Clinical manifestations usually involve nail abnormalities and focal skin lesions such as greasy, brownish-redish keratotic papules, which are distributed in seborrheic areas of the body. Affected individuals are usually heterozygous for mutation in ATPase sarcoplasmic/endoplasmic reticulum Calcium transporting 2 (*ATP2A2*) gene, responsible for encoding Sarco/Endoplasmic Reticulum Calcium ATPase 2 (SERCA2). Although *ATP2A2* mutations significantly reduce ER Ca^{2+} stores in both lesional and non lesional keratinocytes, scientists suggest that a threshold in ER Ca^{2+} depletion is required to disrupt adhesion molecules, as seen in lesional epidermis. Nonetheless, DD hallmarks (acantholysis and dyskeratosis) remain to be fully elucidated. Haploinsufficiency or dominant-negative effect are the postulated pathogenic mechanisms.

Objective This study consists in the molecular characterization of a two-generation Portuguese family with DD history, including co-associated phenotypes and intrafamilial phenotypic variability.

Methods All exons and intron-exon borders of *ATP2A2* were bidirectionally sequenced from DNA and RNA extracted from the five subjects enrolled in this study (four affected individuals and one unaffected individual). Relative levels *SERCA2* mRNA and protein were quantified by RT-qPCR and western blotting, respectively.

Results A splice-site mutation (c.1287+1G>T or IVS10+1G>T) was identified in all four affected individuals, who carry the mutation in heterozigosity, whereas the unaffected individual was shown to carry the wild-type *ATP2A2* sequence in both alleles. This mutation leads to the skipping of full exon 10, which consequently generates a frameshift followed by a premature translation termination codon in exon 11. Results from RT-PCR of a fragment spanning exons 8-13 suggest that a small amount of the mutant transcript escapes NMD and less than 50% expression of the wild-type transcript is detected. In agreement, relative wild-type and mutant *SERCA2* mRNA expression levels assessed by qPCR revealed wild-type gene expression alone revealed a 36% expression, while mutant mRNA expression exhibited

residual levels of 18%. In contrast, western blot results showed 40 to 50% expression of wild-type SERCA2 (115 kDa) in DD patients when compared to the healthy individual, while no sign of the putative mutant SERCA2 (45 kDa) was detected. These findings suggest that despite the escape of a small portion of abnormal *SERCA2* mRNA from NMD, there is still no generation of mutant protein. Therefore, haploinsufficiency appears to be the mechanism underlying DD pathology in these patients. Finally, no genotype-phenotype correlations were found; however, this study contributed to alert physicians to the wide range of defects predicted by *ATP2A2* mutations, including neuropsychiatric features, hearing loss, corneal viral infections by HSV, and glomerular nephritis which is, to the best of our knowledge, here firstly reported in association with DD.

Conclusion As the first study of Portuguese patients, it adds knowledge to the worldwide mutational and clinical spectrum of DD and, importantly, contributes to genetic counseling since it allowed to discard a possible carrier status of the unaffected member of the family.

Keywords: Darier disease; keratosis follicularis; *ATP2A2*; SERCA2; mutational analysis.

Resumo

Introdução A doença de Darier é uma genodermatose rara, de transmissão autossómica dominante, que não distingue sexo ou etnia. As manifestações clínicas caraterísticas desta doença envolvem anomalias nas unhas e lesões cutâneas focais, tais como pápulas queratóticas acastanhadas-avermelhadas distribuídas pelas áreas seborreicas do corpo. Os indivíduos afetados são geralmente heterozigóticos para uma mutação no gene *ATP2A2*, responsável pela codificação da proteína SERCA2. Embora as mutações no gene *ATP2A2* reduzam significativamente as reservas de Ca^{2+} no retículo endoplasmático em células lesionadas e não lesionadas, os cientistas sugerem a necessidade de um limite na depleção destas reservas para interferir no funcionamento de moléculas de adesão, como observado nos queratinócitos acantolíticos da epiderme lesionada. Haploinsuficiência ou efeito dominante negativo são os mecanismos patogénicos postulados para o fenótipo de Darier.

Objetivo Este projeto consiste na caraterização molecular duas gerações de uma família Portuguesa de com histórico de doença de Darier, incluindo co-fenótipos e variabilidade fenotípica intrafamiliar.

Métodos Todos os exões e limites exão-intrão do gene *ATP2A2* foram sequenciados bidirecionalmente a partir de DNA e RNA extraídos dos cinco indivíduos envolvidos neste estudo (quatro afetados e um não afetado). Os níveis relativos de RNA mensageiro e proteína SERCA2 foram quantificados por PCR em tempo real e Western blotting, respetivamente.

Resultados A mutação patogénica (c.1287 + 1G> T ou IVS10 + 1G> T) foi identificada em todos os quatro indivíduos afetados, que exibem a mutação em heterozigotia, enquanto o indivíduo não afetado exibe a sequência normal de *ATP2A2* em ambos os alelos. A mutação altera o *splicing* e leva à eliminação completa do exão 10, que consequentemente altera a leitura da ORF, seguida de um codão de terminação de tradução prematura no exão 11. Os resultados de RT-PCR, correspondente à amplificação de um fragmento abrangendo os exões 8 a 13, sugerem que uma pequena quantidade do transcrito mutado consegue escapar ao NMD e que a expressão de transcrito normal é inferior a 50% relativamente ao indivíduo normal. Em conformidade, os níveis relativos de expressão de RNA mensageiro SERCA2 normal e mutado avaliados por PCR em tempo real revelaram 36% de expressão do gene normal, e aproximadamente 18% de expressão do transcrito mutado. Em contraste, os resultados de

western blot mostraram 40 a 50% de expressão de SERCA2 normal (115 kDa) nos indivíduos com a doença, em comparação com o indivíduo saudável, enquanto nenhum sinal da hipotética proteína mutada (45 kDa) foi detetado. Em geral, os resultados obtidos sugerem que, apesar da pequena porção de transcrito mutado escapar ao NMD, não há tradução em proteína mutada, ou esta deve ser imediatamente degradada. Portanto, a haploinsuficiência parece ser o mecanismo responsável pela patologia de Darier nos doentes estudados. Finalmente, não foi possível identificar qualquer correlação genótipo-fenótipo; no entanto, este estudo contribuiu para alertar os médicos dermatologistas sobre a panóplia de alterações provocadas pelas mutações no gene *ATP2A2*, incluindo sintomas neuropsiquiátricos, perda de audição, infeções virais da córnea causadas por HSV, e nefrite glomerular membranosa que é, pelo que temos conhecimento, relatado neste trabalho pela primeira vez em associação com DD.

Conclusão Como primeiro estudo em doentes Portugueses, esta investigação acrescenta conhecimento ao espetro mutacional e clínico mundial da doença de Darier e, mais importante, contribui para o aconselhamento genético, uma vez que permitiu descartar um possível estatuto de portador do membro não afetado da família.

Palavras-chave: Doença Darier; genodermatose; *ATP2A2*; SERCA2; análise mutacional.

Dedicated to my father, my brother, my uncle, and my cousin…

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Abbreviations

$\alpha^{E}\beta_{7}$	Integrin alpha E beta 7
β-actin	Beta-actin
5-ALA	5-aminolaevulinic Acid
5-FU	5-fluorouracil
ACTB	gene encoding beta-actin
A domain	Actuator domain
ATP	Adenosine triphosphate
ATP2A2	ATPase Sarcoplasmic/endoplasmic Reticulum Calcium-transporting 2
ATP2A2$^{+/-}$	Haploinsufficiency of *ATP2A2*
BAX	BCL2 Associated X
BCL2	B Cell Leukemia/lymphoma 2
BCL-xL	BCL2-like protein 1
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
COX-2	Cyclooxygenase-2
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DCs	Desmosomal Cadherins
DD	Darier Disease
DNA	Deoxyribonucleic Acid
E-cadherin	Epithelial cadherin
ER	Endoplasmic Reticulum
HIV	Human Immunodeficiency Virus
HSV	Herpes Simplex Virus
IgG	Immunoglobulin G
IP_3Rs	Inositol-1,4,5-triphosphate Receptors
MITEs	Miniature Inverted-Repeat Transposable Elements
MN	Membranous Nephropathy
mRNA	Messenger RNA
N domain	Nucleotide-binding domain, also called ATP-binding domain

NMD	Nonsense-Mediated Decay
ORAI1	Calcium Released-activated Calcium Channel Protein 1
P53	Tumor Protein 53
PCR	Polymerase Chain Reaction
Pi	Phosphate group
P domain	Phosphorylation domain
PDT	Photodynamic therapy
P-type	Phosphorylation-type
PTC	Premature translation Termination Codon
qPCR	Quantitative PCR
RAS	Rat sarcoma
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
RyRs	Ryanodine Receptors
SCC	Squamous Cell Carcinoma
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SERCA	Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase
SERCA1	SERCA isoform 1
SERCA2	SERCA isoform 2
SERCA3	SERCA isoform 3
SERCA2a	SERCA isoform 2 splice variant a
SERCA2b	SERCA isoform 2 splice variant b
SERCA2c	SERCA isoform 2 splice variant c
SGPL1	Sphingosine Phosphate Lyase
siRNA	Small Interfering RNA
SOCE	Store-operated Calcium Entry
SPCA1	Calcium-transporting ATPase type 2C member 1
STIM	Stromal-interacting Molecule
TBS	Tris-Buffered Saline
TM domain	Transmembrane domain
TRPC1	Transient Receptor Potential Canonical 1
U1	Small nuclear ribonucleoprotein A
U2	Small nuclear ribonucleoprotein auxiliary factor 35 kDa subunit-related protein 2

U5	Small nuclear ribonucleoprotein 40 kDa protein
U4/U6	Small nuclear ribonucleoprotein Prp3
UPR	Unfolded Protein Response
UTR	Untranslated region
UVB	Ultraviolet B

1. Introduction

This dissertation describes the first molecular characterization of a Portuguese family, in which a rare autosomal dominant disorder, known as Darier Disease (DD, OMIM #124200), segregates. Below, a number of topics will be discussed in order to present a clear background of the disease.

1.1 Historical Aspects and Epidemiology

Darier Disease, also known as Darier-White disease or keratosis follicularis, predominantly affects the skin of individuals regardless their gender or ethnicity [1]. It was discovered in 1889 by Ferdinand-Jean Darier (1856-1938), a French physician, pathologist and dermatologist, and by the American dermatologist James Clarke White (1833-1916), independently [2, 3]. While White described a case of DD, Darier reported a similar case [3]. On one hand, White was the first to suggest the genetic nature of the disease once his patient's daughter started to show similar skin lesions [3]. Darier, on the other hand, was the first to describe the dyskeratotic aspect of the lesional regions in the epidermis [2].

The incidence of Darier disease has been scarcely investigated. According to the statistics, DD's incidence can range from 1 in 30 000 to 1 in 100 000, worldwide. In the British population, the prevalence of the disease is 1 in 55 000, while it is estimated at 1 in 30 000 in Scotland, and 1 in 100 000 in Denmark [4–6]. Despite the current studies about DD's incidence in the North of Europe, an increasing number of mutational analyses and case-reports involving patients from other geographic areas such as Japan, Tunisia, and Taiwan have been described [7–9].

1.2 Epidermis and Calcium Signaling

Representing the largest organ in the human body, the skin provides a primary barrier not only against physical and chemical damage but also against pathogens [10]. The skin comprises three distinct layers: the innermost layer subcutis, the dermis, and the outermost layer epidermis [11]. Epidermis is where keratinocyte differentiation takes place in order to regenerate this skin layer. Keratinocytes are the predominant cells of epidermis, responsible for producing keratin and protecting the skin from chemical and mechanical damage. They proliferate by mitosis in the *stratum basale*, where they are held to each other and to desmosomes of the underlying dermis. After proliferation, keratinocytes differentiate by undergoing major biochemical and morphological changes until they reach the *stratum corneum*, where they finally die and leave the skin surface by shedding [10, 11].

Calcium (Ca^{2+}) plays a major role in all cutaneous layers and is required for the regulation of keratinocyte's proliferation. Throughout epidermis, the Ca^{2+} gradient increases substantially from the *stratum basale* to the *stratum granulosum*, where it reaches its peak, and then drops bluntly to zero in the *stratum corneum*, which is made of dead keratinocytes (Figure 1) [12]. In the presence of deregulated intracellular Ca^{2+} levels, the epidermal stratification is desynchronized and the texture of the skin becomes abnormal [10], which has been observed in skin disorders, such as Darier Disease, where endoplasmic reticulum (ER) Ca^{2+} stores happen to be depleted [13].

The controlled Ca^{2+} release from the ER Ca^{2+} stores creates the luminal environment needed for crucial biological activities such as protein folding [14], cellular proliferation, apoptosis, and differentiation [15], and excitability and synaptic plasticity in neurons [16]. To function as a dynamic Ca^{2+} pool and allow immediate electrical or chemical signaling events in neurons, the ER must express three distinct types of proteins [14]. These proteins include Ca^{2+} pumps that actively promote Ca^{2+} transport from the cytosol to the lumen of the ER, known as Sarcoplasmic/Endoplasmic Reticulum Calcium ATPases (SERCAs), inositol-1,4,5-triphosphate receptors (IP_3Rs) and ryanodine receptors (RyRs) which control the release of ER Ca^{2+} to the cytosol along its electrochemical gradient.

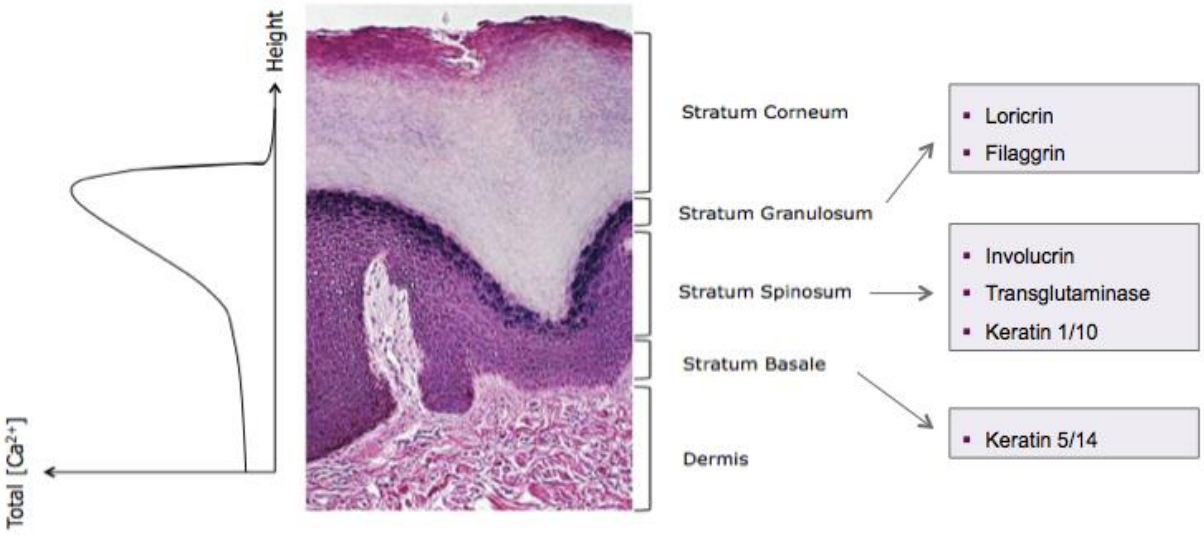


Figure 1 | Intracellular and extracellular calcium concentration profile and differentiation markers along the epidermal strata (X100 Hematoxylin and Eosin staining) (https://basicmedicalkey.com/skin-6/#ch18lev9) [11, 12].

To prevent compromising the normal function of the ER, whenever stress is triggered by a disruption in Ca^{2+} homeostasis, an unfolded protein response (UPR) is initiated. If the stress is either consistent or too severe, the UPR will not be strong enough to overcome it [14]. Consequently, apoptosis is induced to eliminate the damaged cell, which usually occurs in DD and neurodegenerative diseases [14].

In normal conditions, there are molecular mechanisms that help preventing ER Ca^{2+} depletion or overload. Ca^{2+} release from the ER in response to physiological stimuli evokes mitochondrial activity, leading to the increase of ATP synthesis, and to an initial deficit of Ca^{2+} concentration in the ER. Stromal-interacting molecule (STIM), a protein localized in the ER membrane, senses this deficit and interacts with the plasma-membrane and Ca^{2+} channel ORAI1 (calcium released-activated calcium channel protein 1), enabling store-operated Ca^{2+} entry (SOCE) [13, 14, 17]. Hence, while Ca^{2+} is being released from the ER, there will be more Ca^{2+} entering the cell. Once SOCE increases cytosolic Ca^{2+} levels, SERCA proteins use ATP to actively pump calcium into the ER, in order to reduce cytosolic Ca^{2+} levels and restore ER Ca^{2+} levels, allowing ER-related processes to continue properly [14].

Under pathological conditions, impairment in SERCA activity is sufficient to compromise steady-state Ca^{2+} levels in the ER, along with its functions, leading to ER Ca^{2+}

depletion. The importance of ER calcium stores controlled by Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase 2 (SERCA2) was first revealed by a lethal phenotype of SERCA2 knockout mice, whose ER calcium levels were increasingly depleted [18]. *In vitro* experiments, where normal epidermal cell lines under inhibition of SERCA2 were cultured with low calcium media and then switched to high calcium media, have also shown the importance of SERCA2-gated Ca^{2+} stores [19]. Nevertheless, it appears that the human body has its ways to compensate for SERCA2 loss, as can be observed in most DD patients.

1.3 Gene and Protein

The inherited character of Darier disease relies on a heterozygous mutation in the ATPase sarcoplasmic/endoplasmic reticulum Ca^{2+} transporting 2 (*ATP2A2*) gene, which encodes the SERCA2 protein. *ATP2A2* mutations lead to the depletion of ER Ca^{2+} stores, particularly in keratinocytes since these are frequently exposed to ER stressors such as UVB light [1, 13]. Located in chromosome 12q23-24, *ATP2A2* is transcribed into 21 exons and alternative splicing of the resulting pre-mRNA generates three major isoforms: SERCA2a, SERCA2b, and SERCA2c [1, 20]. Alternative splicing of exon 20 leads to the production of SERCA2a and SERCA2b isoforms, while the inclusion of a short intronic sequence containing an in-frame STOP codon between exons 20 and 21 of SERCA2a originates SERCA2c [13, 20].

SERCA2, along with SERCA1 and SERCA3, belong to P-type superfamily of Ca^{2+} pumps that can be detected in the majority of eukaryotic cells' sarcoplasmic/endoplasmic reticulum. These pumps share a highly conserved general structure, which consists of three cytosolic domains – actuator domain (A), nucleotide-binding domain (N), and phosphorylation domain (P) – and a transmembrane domain (TM), where Ca^{2+} binding sites are located (Figure 2) [15]. These pumps also share the same reaction cycle which starts with the binding of two calcium ions to the Ca^{2+} binding sites that are facing the cytoplasm, followed by the binding of one ATP molecule to the N domain and posterior hydrolysis by P domain, leading to the phosphorylation of a highly conserved aspartic acid residue (Asp351) (Figure 3) [15, 21]. This auto-phosphorylation allows the pump to switch conformation so its Ca^{2+} binding sites can face the luminal space and release the calcium ions into the ER. The

pump is regenerated to its initial state through dephosphorylation of the P domain, catalyzed by H_2O and the conserved glutamate in the A domain [15, 21].

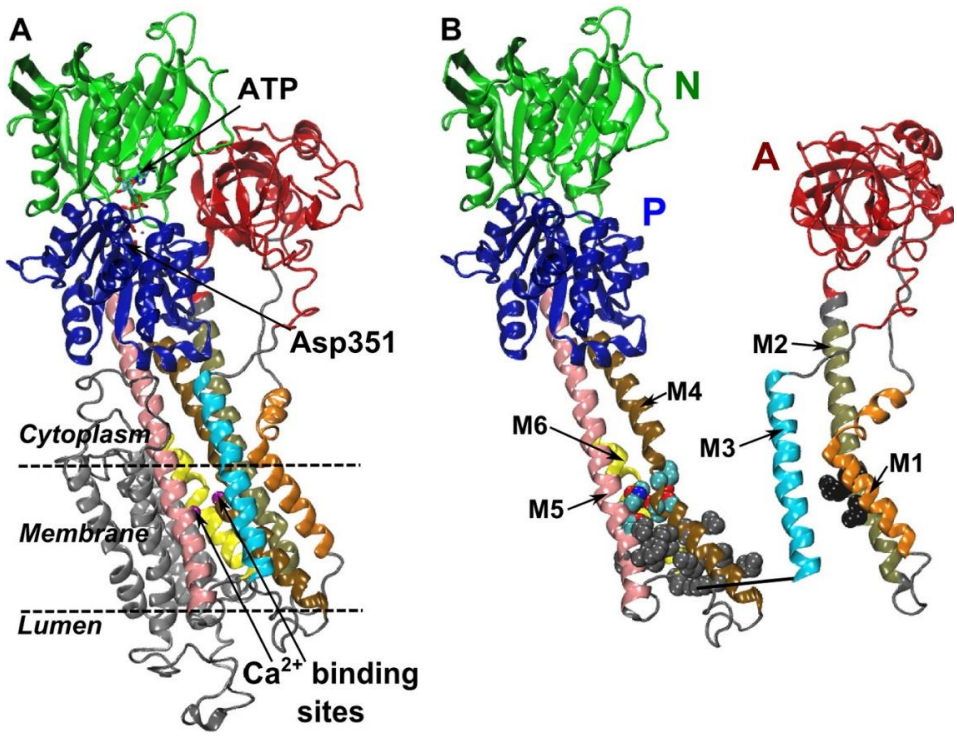


Figure 2 | Overall topology of the P-type ATPase calcium pump SERCA. **(A)** The cytoplasmic headpiece comprises the nucleotide binding (N), phosphorylation (P) and actuator (A) domains. The ATP binds to the N domain. The conserved aspartic acid that gets phosphorylated is in the P domain. The calcium binding sites are located in the transmembrane (TM) domain. The TM domain is made of ten TM helices (labeled M1 to M10 or 11). **(B)** The A domain is covalently linked to the helices M1, M2 and M3, and the P domain is covalently linked to the helices M4 and M5. Adapted from [22].

Furthermore, several studies have demonstrated, through radiation inactivation analysis and optical diffractometry, that these ATPases appear to be organized as dimers in the sarcoplasmic/endoplasmic membrane [23–26]. Nevertheless, it has not been proved whether monomers are not sufficient for normal function of the pump.

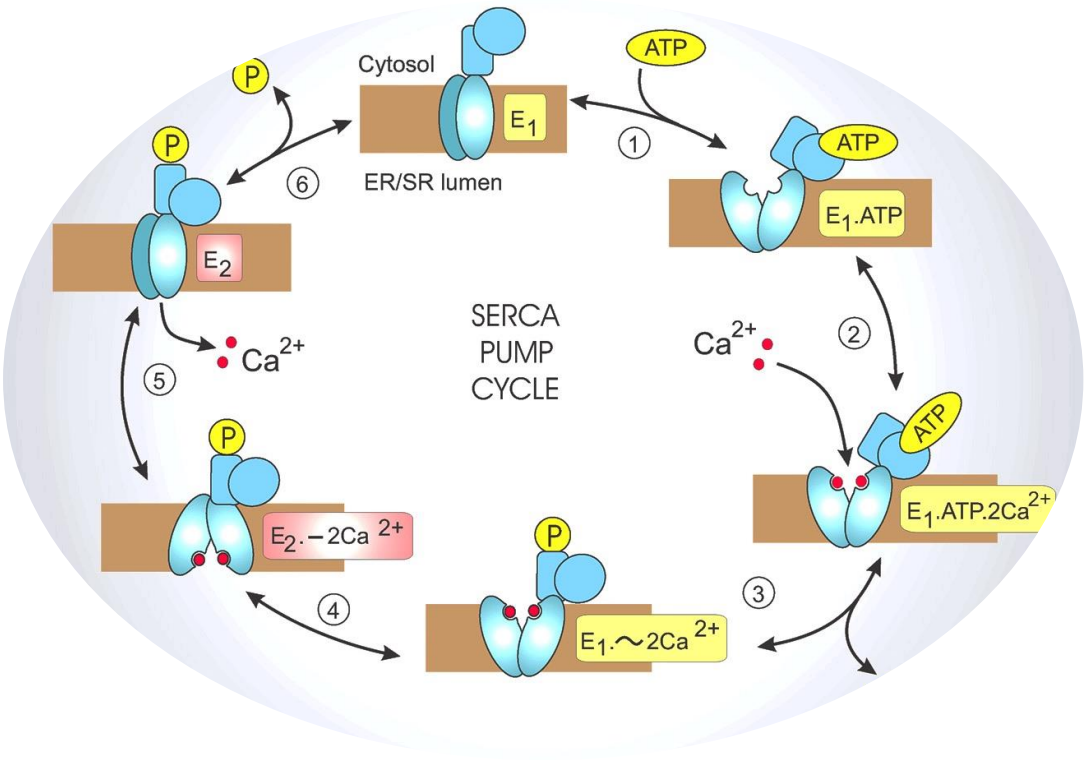


Figure 3 | Reaction cycle for the Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase (SERCA) pumps. During this cycle, a series of biochemical reactions lead to two major conformational states of the Ca^{2+} pump: an E_1 state when two high-affinity Ca^{2+} -binding sites that are facing the cytoplasm become saturated, and an E_2 state where the Ca^{2+} -binding sites have a low-affinity and therefore switch to the opposite side and Ca^{2+} is released to the lumen. During each cycle, two Ca^{2+} ions are pumped for each ATP hydrolyzed. (Adapted from http://www.cellsignallingbiology.org/csb/005/csb005fig5_SERCA_pump_cycle.htm?resolution=HIGH).

Despite their similarities, these pumps do not share a common expression pattern, and neither do SERCA2 splice variants. SERCA2a (997 amino acids) has been found to be restrained to cardiomyocytes, cardiac slow-twitch muscle, skeletal and smooth muscle, pancreatic, and cerebellar Purkinje neurons (http://www.uniprot.org/uniprot/P16615) [13]. It is expressed at lower levels in the epidermis and has a two-fold lower affinity for Ca^{2+} and a two-fold higher catalytic turnover rate, when compared to SERCA2b [27]. The reduced expression of isoform SERCA2a, found in animal models of heart failure and in humans with heart failure, confirms its essential role in platelet and cardiac function [28]. In fact, in healthy individuals, SERCA2a is usually expressed at elevated levels in the heart and represents a key player in cardiac myocyte Ca^{2+} regulation required for excitation contraction coupling [28].

Mutations in the *ATP2A2* gene affecting SERCA2a levels should therefore provoke an augment in platelet aggregation and cardiac dysfunction. However, there is a clear paradox since DD patients have been shown to have a normal cardiac function and platelet activity [28]. Researchers suggest that SERCA3, which is also present in platelets, may compensate for the platelet SERCA2a deficit in DD patients [29].

SERCA2b (1042 amino acids) is the ubiquitous housekeeping isoform, expressed in non-muscle cells and highly expressed in epidermal cells [1, 29]. Comparing to SERCA2a, isoform b contains an additional transmembrane domain, encoded by exon 21, and a tail known as 2b tail, which extends to the lumen of the ER. Interestingly, SERCA2b has the highest affinity for Ca^{2+} among all splice variants [27]. Moreover, a mutation in exon 21, affecting only SERCA2b isoform's expression appears to be enough to cause DD, suggesting the crucial role of this pump in the skin and the inability of other splice variants to compensate the loss of SERCA2b normal function [13]. Nevertheless, it has been proposed the existence of putative compensatory mechanisms in DD keratinocytes, such as secretory pathway Ca^{2+}-ATPase pump type 1 (SPCA1), that may help overcoming the intracellular [Ca^{2+}] imbalance caused by mutations in the *ATP2A2* gene [30].

The latest isoform described, SERCA2c (999 amino acids), is expressed in epithelial, mesenchymal and hematopoietic cell lines and monocytes [24, 28]. This isoform shows a Ca^{2+} affinity and catalytic turnover rate similar to that of SERCA2a and SERCA2b, respectively [20].

According to Leiden Open Variation Database v3.0 (http://www.lovd.nl/3.0/home), 420 variants have been reported in the *ATP2A2* gene. DD mutations are mostly private ones (Figure 4). The majority of these private variants are missense mutations, which have been reported to be associated with a more severe phenotype [13, 31]. Mutations yielding a premature stop codon, nonsense, and splice-site mutations, and in-frame deletions and insertions have also been reported in DD patients [13]. Depending on the protein domain affected by these mutations, one may suggest a series of functional disruptions, including loss of Ca^{2+} affinity, obstruction of Ca^{2+}-binding sites, decreased ATP affinity, effects in phosphorylation by ATP and Pi, effects on conformational shifts, inhibition of dephosphorylation, or disassembling of Ca^{2+} transport from ATP hydrolysis [32].

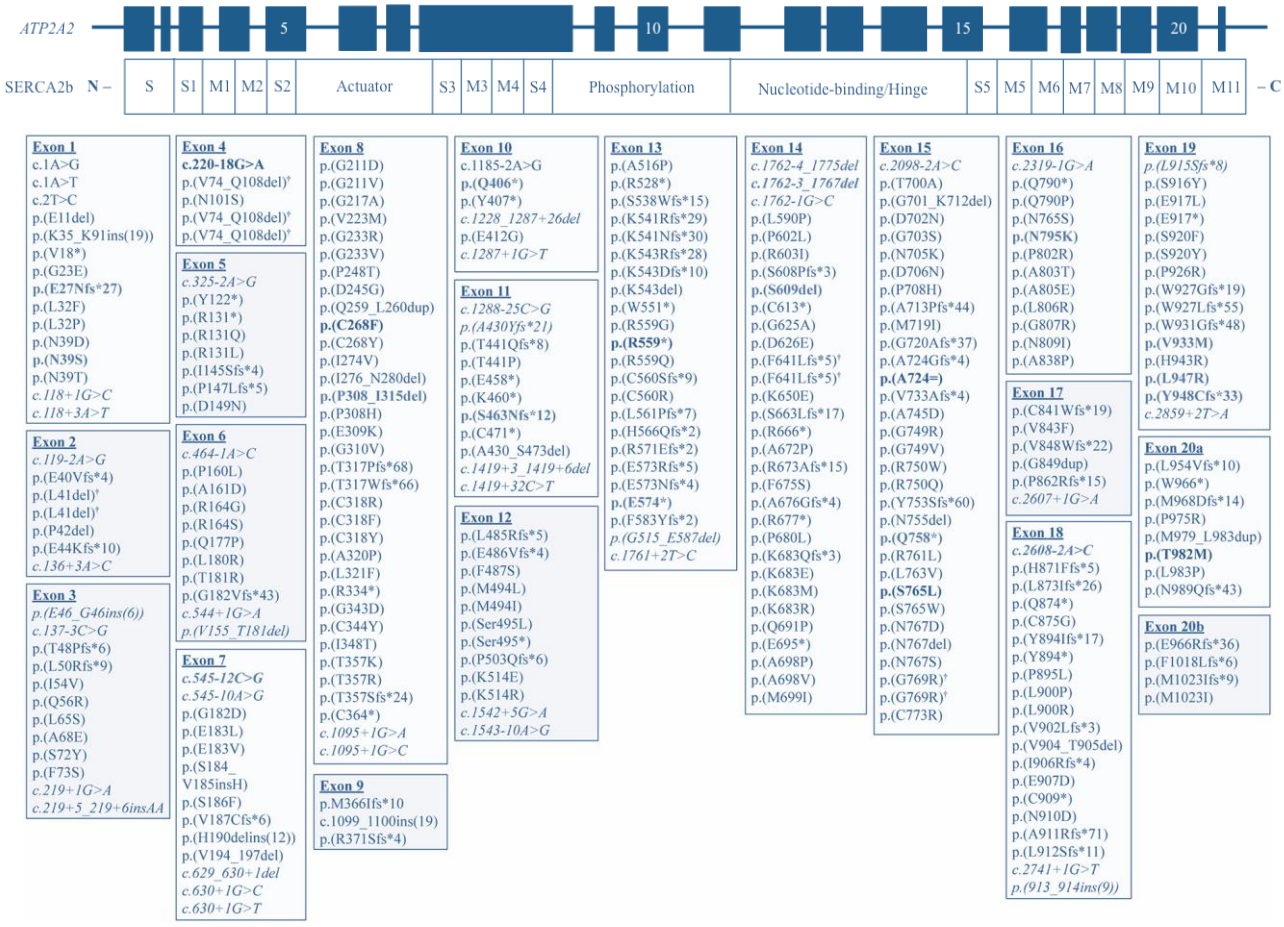


Figure 4 | Schematic representation of *ATP2A2* gene and encoded protein SERCA2 isoform b and spectrum of *ATP2A2* variants responsible for DD. All 21 exons and respective protein domains are represented. S- Stalk domain, M – Transmembrane domain. Exons 5, 10, 15, and 20 are numbered in the *ATP2A2* gene representation. The spectrum of *ATP2A2* variants below the gene and protein schemes refer to exon 20 as "20a" and exon 21 as "20b" [33].

1.4 Clinical Manifestations

DD runs a chronic and relapsing course after its onset and may aggravate with age [5, 13]. The onset of the phenotypic abnormalities is usually during the first or second decade of life; however, delayed onset may happen as it has been reported [4, 6]. The disease is characterized by nail abnormalities and focal skin lesions such as greasy, skin-colored (brown or yellow) keratotic papules and eruptions (Figure 5) [1, 34]. The majority of skin lesions are distributed in seborrheic areas of the upper back, folds, neck, scalp, chest, and face. These lesions become easily infected, leading to major discomfort and exacerbation of the condition. Once infected, there is a high prevalence of *Staphylococcus aureus* colonization in these lesions [35]. Furthermore, external factors such as UVB radiation, heat, friction, sweat, and humidity play a key role in the worsening and relapse of skin lesions [1, 13, 34]. The nail phenotype consists of longitudinal splitting with V-shaped notches at the free margin of the nail, and subungual hyperkeratosis [1]. Palmoplantar pits are quite common, and hyperkeratosis of palms and soles may also occur. Moreover, the oral mucosa and gingiva may also suffer from lesions [1]. However, the above-mentioned clinical manifestations vary in severity from patient to patient and even within affected members of the same family.

1.5 Histopathology

Although genetic testing is the most accurate method to diagnose a genetic disorder, the diagnosis of DD is usually done by histological examination of a skin biopsy [36]. Histologically, two hallmarks of DD can be identified in focal epidermal lesions: (1) acantholysis, which consists of loss of cell-to-cell adhesion and suprabasal clefting as a result of desmosomal disintegration; (2) dyskeratosis, characterized by abnormal/premature keratinocyte differentiation and apoptotic keratinocytes called “corps ronds” with a central basophilic and pyknotic nucleus surrounded by a clear halo (Figure 6) [1, 42]. Acantholysis can be observed in more detail by electron microscopy analysis, which demonstrates an impaired number of desmosomes and desmosomal cadherins [5, 43]. Desmosomes are intercellular junctions, composed by adhesion molecules such as cadherins and desmosomal plaque proteins, responsible for connecting adjacent cells. These cellular structures are

essential in mammals' cardiac and epithelial tissues since these are subject to mechanical stress [39]. Therefore, disruption in the stability and cell surface expression of desmosomal proteins contributes to acantholysis since it impairs cell-to-cell adhesion in lesional keratinocytes. Additionally, cadherins are known for their important role in neuronal functions including the formation of specific neural circuits [40].

Figure 5 | Clinical manifestations of Darier disease patients. A Reddish, greasy, crusted papules on the abdomen. Adapted from [41]. **B** Confluent reddish-brownish papules in the seborrheic areas of the chest, submammary areas, and arms. Adapted from [42]. **C** Papular lesions cover skin in the occipital area of female patient with Darier's disease. Adapted from [43]. **D** Nail involvement in Darier's disease. Adapted from [1]. **E** Involvement of lower limbs with marked keratosis and fissures, which may lead to secondary infection in a patient with extremely severe condition. Adapted from [44]. **F** Papillomatous and macerated lesion in the groin. Adapted from [45].

Studies, where authors used thapsigargin as a specific inhibitor of SERCA or small interfering RNA directed to SERCA2, have provided evidence for the intrinsic role of SERCA2 in the disruption of intracellular Ca^{2+} signaling and consequent mislocalization of desmosomal cadherins [19, 34, 46, 47]. Nevertheless, the intracellular Ca^{2+} role in the regulation of adhesion molecules needs further investigation, as well as the role of cadherins in acantholysis and dyskeratosis in lesional keratinocytes.

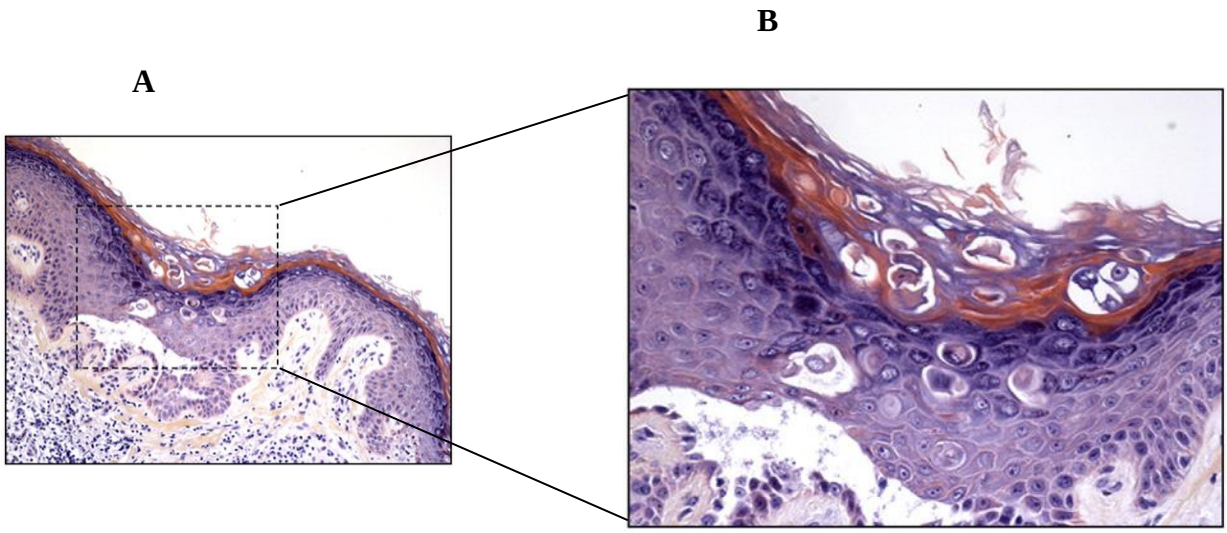


Figure 6 | Histological section of Darier disease skin. Skin biopsy from Darier disease where a suprabasal cleft of the epidermis formed by acantholytic cells can be observed, as well rounded dyskeratotic cells with a dark nucleus, so called "corps ronds", and hyperkeratosis. B is an enlargement of A. Adapted from [13].

1.6 Associated Manifestations

Although skin lesions represent the major pathophysiological outcome of mutations in the *ATP2A2* gene, additional clinical manifestations appear to be linked to DD, being neuropsychiatric features the most common co-occurrences.

1.6.1 Neuropsychiatric manifestations

Interestingly, the impairment of Ca^{2+} homeostasis and connectivity defects due to cadherin disruption are involved in cognitive and psychiatric disorders [45, 48, 49]. In fact, a range of neuropsychiatric features such as depression, suicidal behavior, bipolar disorder, schizophrenia, intellectual disability, epilepsy, cognitive deficit, mental retardation, and learning disability have been reported in DD patients [48–51]. A study involving unrelated DD patients revealed that 31% of these have a lifetime history of suicidal thoughts and 13% have attempted suicide, which did not have a relationship with DD or worsening of the disease [52]. Gordon-Smith *et al.* have found higher lifetime prevalence rates of mood disorders (50%), especially major depression (30%) and epilepsy (3%), to be present within a sample of one hundred unrelated DD patients compared with that in the general population [52]. In agreement with these findings, Celik *et al.* highlighted that the overall prevalence of epilepsy is 30–42.9 in 1000 DD patients, while the reported lifetime prevalence rates of epilepsy in the general population are variable, with a percentage of 1.3% [53].

Mutational analysis of a number of DD patients with neuropsychiatric phenotypes has shown predominance of missense mutations affecting the ATP-binding domain, between exons 13 and 19 of the *ATP2A2* gene, whereas patients with DD alone have demonstrated a more diverse series of mutations predominantly spread between exon/intron 1 and 19 [32]. Therefore, it is suggestible that missense mutations in the ATP-binding domain turn out to be crucial contributors for abnormalities in neuron signaling. Nevertheless, the reason why psychiatric phenotypes are not co-segregated among every family member with the same DD mutation remains to be unraveled.

Summing up, there are four reasons why these co-occurrences might be present in DD patients: (1) a predisposition of *ATP2A2* gene mutation towards the neuropsychiatric illness; (2) *ATP2A2* gene location near a susceptibility gene for the neuropsychiatric illness; (3) pleiotropic effects of SERCA2 since it is expressed in both epidermal and neuronal cells; or (4) by pure chance, given that the mentioned psychoses show a high prevalence worldwide [50].

1.6.2 Other manifestations

Besides neuropsychiatric phenotypes, there are other potential associated manifestations in DD that should keep dermatologists alert, such as renal impairment, testicular agenesis, cataracts, skin cancers, bone cysts, and corneal and ocular sequels [54–57]. Some researchers even point out for the genetic nature of DD as a model of skin carcinogenesis since the disruption of Ca^{2+} homeostasis, among other disturbances, is capable of inducing several genetic alterations, as it has been observed in cutaneous tumorigenesis models [58]. Opposite to the major mechanism of cancer, in *ATP2A2*$^{+/-}$ mice, neither RAS nor P53 proto-oncogenes were mutated, suggesting a novel form of cancer susceptibility through mutated SERCA2 [59]. This evidence is in agreement with a case report about a DD patient who had no history of smoking, drugs, or familial history of cancers, and still developed a squamous cell carcinoma (SCC) in the same area of a skin lesion, suggesting that SCC and DD may share common molecular disruption mechanisms [58].

Increasing literature gives more and more evidence that these secondary manifestations are unlikely to occur by mere chance in DD patients. However, further research is needed to clarify the mechanisms underneath these co-phenotypes and their genetic predisposition.

1.7 Intrafamilial phenotypic variability

The incidence and severity of skin lesions are not equally observed from parent to child, or sibling to sibling, who carry the very same pathogenic mutation in the *ATP2A2* gene. Furthermore, despite the linkage between neuropsychiatric phenotypes and DD, the formers are not co-segregated in every affected member of the family. Researchers highlight for the presence of nucleotide alterations in the *ATP2A2* gene, apart from the pathogenic mutation, as a potential cause for such clinical variability between relatives [30]. Although phenotypic variability can be observed in several families that segregate the disease, linkage studies embracing this matter are scarce and well-defined genotype-phenotype correlations have not yet been achieved.

1.8 Mechanism of Action

Throughout literature, haploinsufficiency or dominant-negative effect have been the postulated pathogenic mechanisms of DD, which is characterized by a heterozygous mutation in the *ATP2A2* gene in most patients. According to haploinsufficiency, a single wild-type allele is not able to provide enough amount of protein and thus does not allow normal cell viability or function. However, if haploinsufficiency ought to be enough to cause DD, then why is the onset during the first or second decade of life rather than at birth? Why does DD lead to focal lesions rather than generalized dermatoses? How can SERCA2 haploinsufficiency explain the variable clinical severity among family members who carry the same mutation? All these observations have led scientists to further investigate the effect of DD mutations and/or SERCA2 inhibition.

Increasing *in vitro* studies involving epidermal and other cell types treated with SERCA-specific inhibitors, siRNA directed to *SERCA2* mRNA, or site-directed mutations in *ATP2A2* gene have demonstrated that SERCA2 haploinsufficiency may not be enough to cause DD [19, 60, 61]. SERCA2 mutants studied in neuronal and epidermal cell lines revealed to be insoluble or partially soluble due to their misfolded state, intrinsic to the mutation itself [60]. Instead of being degraded by proteasomes, the insoluble SERCA2 mutants have shown to gather around the nucleus, originating aggregates that contribute to the rounding up of cells seen in DD epidermis [60]. On the other hand, the perinuclear aggregation of these proteins could rely on the ability of SERCA2 mutant monomers to interact with the wild-type encoded SERCA2 in order to form dimers, as it has been demonstrated by co-immunoprecipitation of SERCA2 mutants and wild-type proteins from human embryonic kidney cells co-transfected with both SERCA2b plasmids [61]. Ahn *et al.* suggest that different *ATP2A2* mutations may lead to different SERCA2 mutants, which may be responsible for the different phenotypes in DD patients according to the mutation's impact on the activity of the wild-type encoded pump. This study reveals that most mutants not only interact with wild-type SERCA2 to form a dysfunctional dimer but also inhibit the rate of Ca^{2+} uptake by wild-type SERCA2, causing a dominant-negative effect [61].

Interestingly, it has been demonstrated that both lesional and non-lesional DD keratinocytes suffer ER Ca^{2+} depletion, but only lesional keratinocytes exhibit mislocalization and altered trafficking of desmosomal cadherins (DCs) [19, 34]. This aspect of DD's pathology was enlightened by Li *et al.* who revealed that inhibition of SERCA2 by

thapsigargin in human epidermal keratinocytes leads to the reduction of ER Ca^{2+} levels below a threshold level, which consequently causes ER retention of nascent DCs [19]. Furthermore, it has been shown that the maturation (ER-to-Golgi transport) of nascent DCs is not affected when SERCA2 activity was inhibited by siRNA, suggesting that haploinsufficiency does not cause enough ER Ca^{2+} depletion to disrupt adhesion in DD pathology [19]. Presenting similar results, Celli et al. have reported that treatment of normal cells cultured with thapsigargin can disrupt the assembly of desmoplakin and E-cadherin to cell-to-cell boarders in a dose-dependent manner [34]. However, since thapsigargin not only blocks SERCA2 but all other SERCA pumps' activity, it would be worth to further investigate the assembly of cadherin molecules in SERCA2 mutant cells.

Another hallmark of DD is the abnormal differentiation that occurs in lesional keratinocytes, including hyperproliferation and apoptosis of these cells. It is not surprising that once Ca^{2+} profile along the epidermis is disrupted, Ca^{2+} dependent activities such as differentiation, proliferation and apoptosis become deregulated. Calcium signaling may as well be crucial not only to the differentiation markers in each epidermal stratum, but for the BCL2 family which is linked to apoptosis. Bongiorno *et. al* have found decreased levels of BCL2 and BCL-xL, and deregulated levels of BAX in histological specimens of DD [62]. These results may explain the presence of "corps ronds" in histological sections in lesional epidermis. In addition, BCL-2 was shown to upregulate and possibly interact with SERCA pumps in order to preserve and stabilize ER Ca^{2+} pools, which is one of the ways that BCL-2 protects cells from apoptosis [62]. These findings suggest that (1) once there is not enough SERCA2 localized in the membrane of the ER, BCL-2 is unable to interact with SERCA2 in order to maintain ER Ca^{2+} stores, which consequently subjects keratinocytes to apoptosis; (2) with the presence of deregulated BAX, BCL-2 is also deregulated; (3) a decreased level in BCL-xL may also contribute to apoptosis in keratinocytes and to abnormal mitotic process; and finally (4) the imbalance of these three apoptosis-regulator proteins may underline the apoptotic pathway responsible for dyskeratosis seen in DD [62]. Nonetheless, DD hallmarks remain to be fully elucidated.

1.9 Treatment

Heretofore there has not been found a treatment whose efficacy does not vary among DD population. Personal hygiene, the use of sunscreen and avoidance from solar exposure, heat, high humidity, and tasks that could lead to excessive sweat are some of the indispensable measures in the day life management of the disease [44]. Currently, oral or systemic retinoids are the most frequently prescribed drugs to manage DD. In some cases, these drugs are able to reduce hyperkeratosis and flatten the papular component of the skin eruptions effectively. However, in most cases, retinoids are associated with a large number of noxious side effects, including mucosal dryness, itching, and severe or worsening of psychiatric symptoms [39, 63, 64]. In addition, most retinoids cannot be prescribed to women of child-bearing age [41, 65].

To date, the conventional treatments (retinoids, emollients and corticosteroids) used to manage DD's cutaneous manifestations have yield disappointing results in general. Efficacy, safety, and long-term absence of relapses are crucial factors for a successful treatment. Therefore, other alternative drugs and therapies have recently been tested in DD patients.

A promising retinoid is the vitamin A derivate drug, alitretinoin (9-cis retinoic acid), which has shown cutaneous improvement due to its anti-proliferative and immune-modulatory effects on keratinocytes, and absence of relapse or relevant side effects within 15 months [66]. As opposed to other retinoids, alitretinoin's short half-life suggests that it could be used to treat females of childbearing age as well [66]. Abe et al. reported a great clinical response and safety of vitamin D3 analog, Tacalcitol, in a DD patient [63].

Furthermore, therapies used in cancer patients, such as photodynamic therapy and 5-fluorouracil (5-FU), are also being tested in DD patients with benign or malignant skin lesions. Promising reports on treatment with topical 5-FU reveal that this compound is well tolerated by the majority of patients, who revealed a continuous decrease in cutaneous inflammation, and relief of burning and itching sensations caused by the condition of the disease [67].

In a limited number of patients, photodynamic therapy (PDT) has been reported to be tissue selective and responsible for successful treatment of DD [65, 68]. PDT consists on the combination of a photosensitizer, usually topical 5-aminolaevulinic acid (5-ALA), which accumulates in the target cells, an activating light source, and oxygen which cause a photo-oxidative reaction within the lesional tissue [65]. As a result, reactive oxygen species are

generated, inducing apoptosis and necrosis of target cells. Unlike dermabrasion and laser therapies, PDT prevents the risk of scarring and relapse of the condition due to tissue selectiveness [65].

The compounds and therapies above-mentioned are excellent candidates to manage DD. The increasing promising reports involving single patient or small number sample trials have opened doors to possible wider trials in the future. Randomized clinical trials would be the best way to confirm the effectiveness and safety of these recently tested drugs [67].

Additionally, there have been reported promising targets for DD, including sphingosine phosphate lyase (SGPL1), Transient Receptor Potential Canonical 1 (TRPC1), and cyclooxygenase-2 (COX-2), whose modulation could be a potential treatment for managing abnormal differentiation and loss of intercellular adhesion, keratinocyte proliferation, or for the enhancement of SERCA2 expression, respectively [34, 69, 70].

Despite the progress in gene therapies for heritable skin disorders, there is a considerable need to target DD by correcting the underlying mutation in somatic cells, either by using interfering RNAs, adeno-associated virus, or even CRISPR [71, 72].

2. Aims of the study

This project consists in the molecular characterization of a two-generation Portuguese family with DD history, including co-associated phenotypes and intrafamilial phenotypic variability. Accordingly, there are four aims that must be considered:

Aim no. 1: Identify and describe the pathogenic mutation that is causing the pathology in the family.

Aim no. 2: Examine whether the unaffected individual is a carrier but due to possible incomplete penetrance or later onset of the disease does not yet show clinical manifestations.

Aim no. 3: Analyze the pathogenic mutation's effect at the transcriptional and translational levels.

Aim no. 4: Investigate genotype-phenotype correlations in an attempt to explain clinical heterogeneity of the affected family members by coupling the results from mutational analysis to their clinical history.

The results from this mutation analysis will determine whether the patients and possible carrier of the mutation should benefit from genetic counseling. Questions such as "Is this disease going to affect my children one day?", "What should be monitored in my future children?" can eventually be brought up to surface and genetic counseling will help by inform them tightly about the risk of their progeny developing DD and co-occurrences, when planning or expecting to have a baby.

3. Participants and Methods

3.1 Case Report

Five individuals from the two-generation family agreed to enroll in this study: four affected relatives (I.2, I.3, II.1 and II.3) and one unaffected relative II.2 (Figure 7). Relatives I.1 and I.4 were not considered to integrate the following study since they do not show any clinical history of the disease.

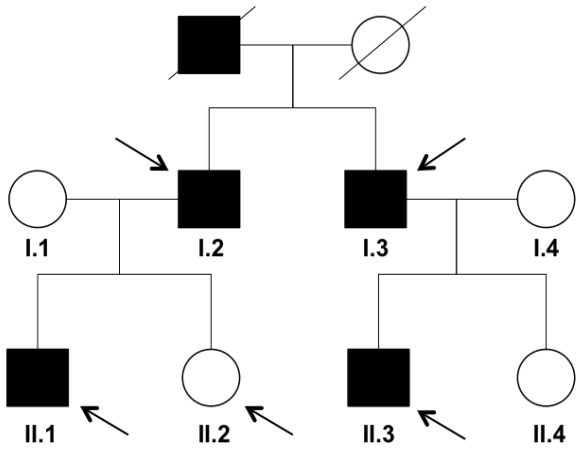


Figure 7 | Pedigree of the two-generation family. The affected individuals are denoted by solid black symbols whereas unaffected individuals are denoted by open symbols. Arrows point to the individuals who participate in this study.

All affected family members were diagnosed by dermopathology during their first decade of life. These individuals share a monomorphic dermatosis, notably symmetrical, containing multiple non-follicular hyperkeratotic papules with 2-4 mm in diameter, brownish, rounded and occasionally crusted, on skin usually healthy or erythematous skin during periods of inflammatory exacerbation. The lesions are distributed along the entire lateral cervical region, anterior thorax and lower abdominal region (especially flanks), appearing

also in the scalp, and predominant in retroauricular region. Palms of the hands present multiple millimetric depressions filled by keratin (palmar pits). Nails of the hands and feet present discrete longitudinal leukonychia, fragility, fissure and dyskeratosis with V-shaped notches on the distal end of the nail plate. Eventual relapses of greater clinical severity usually lead to skin eruptions or infections by bacterial agents. Patients clinical course is in agreement with the worsening of the disease over the years. Moreover, patients agree that skin lesions are exacerbated by sweat, friction, and sunlight, particularly during the summer.

However, the clinical severity of the disease varies from patient to patient. At the skin level, patient I.1 has more relapses than his brother (I.3) while patient II.1's relapses are more frequently and more severe than those of his father (I.1). In addition to their skin phenotype, viral, immunologic, and neuropsychiatric features occur in most patients (Table 1).

To manage DD, the patients take medicines prescribed by dermatologists such emollients, topical antibiotics, and retinoids such as acitretin, a derivative of vitamin A. They also avoid being exposed to the sun during the summer in order to prevent focal lesions to emerge. Patients with other occurrences, referred in Table 1, take additional medicines to manage these.

Table 1 | Additional information about participants' actual age, age of onset, and other phenotypic manifestations.

Individuals	Actual Age	Age of DD onset	Neuropsychiatric occurrences	Other occurrences / age of onset
I.2	61	1st decade of life, around six years old	None	Herpes simplex virus in the left eye / 39
I.3	56		None	None
II.1	31		Single suicide attempt Depressive moods	Membranous glomerulonephritis / 21 Left ear deafness
II.3	34		Epilepsy	Leukemia
II.2	24	NA	NA	NA

NA – not applicable

3.2 Sample collection

Peripheral blood was collected by venipuncture from the enrolled individuals and written informed consent was obtained from each one. In the case of the affected individuals that are followed by a dermatologist, respective doctors were informed about the study. Blood samples were used for the extraction of nucleic acids and preparation of protein homogenates.

3.3 DNA and RNA extraction

Lymphocytes from a total peripheral blood volume of 14 mL from each subject were freshly isolated in a Ficoll density gradient and each pellet was stored at -80ºC before further use. Genomic DNA and total RNA were extracted from peripheral blood lymphocytes using Citogene ® DNA Blood Kit (Citomed) and Isol-RNA Lysis Reagent (5PRIME), respectively. The extraction of genomic DNA was performed based on a standard protocol involving cellular lysis, followed by protein precipitation, and DNA hydration. Total RNA extraction was performed according to the TRIzol method using chloroform to separate RNA from DNA and contaminants, followed by isopropanol precipitation, and pellet solubilization in RNase-free water. DNA and RNA concentrations and A_{260}:A_{280} ratio from each sample were assessed using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). Quality and integrity of nucleic acids were also examined by 1% agarose gel electrophoresis. Extracted DNA samples were preserved at 4ºC before further use, while total RNA samples were stored at -80°C.

3.4 PCR and RT-PCR amplification

Polymerase chain reaction (PCR) amplification of all 21 exons and flanking intronic boundaries of the *ATP2A2* gene was performed using 17 sets of primers, specifically designed with Primer3 program (http://primer3.ut.ee) (Table 2).

Table 2 | PCR primers and related parameters for amplification of *ATP2A2* from genomic DNA.

Exon	Forward Primer	Reverse Primer	Coordinates[a] (Forward; Reverse)	Product size (bp)	Annealing Temperature (ºC)
1	gcaagaggaggaggggaga	ccatcttccctggctctccc	5035-5053; 5322-5341	307	58
2+3	cctccctcttgacacattgct	agacacagcttgcaactcca	5934-5954; 6268-6287	354	
4	catgttgggcaggttggtct	ccattgcactccagcctg	15221-15240; 15683-15700	480	
5	tgtccttgtgtctgttgcct	tgacaggaagggaggtgcta	19810-19829; 20280-20299	490	
6	ctcatttcagccgcctttt	aagggacagtgaggcaagag	46332-46349; 46514-46533	202	
7	ggtgggcatgaatgagaggt	agtgatggttggcagtgaaa	49686-49705; 49915-49934	249	
8	cagcgtcggtatttaagttggg	acaaagaaccacgacacgga	50862-50883; 51421-51440	573	
9	tgtttgcctttgtcctaagct	tgccacaccagatcctttaaa	55876-55896; 56080-56100	225	
10	ggggcgggaggaatcaatag	ctttcattccacccacccca	56492-56511; 56706-56725	234	
11	gacagattgtgcttttgtgga	gagagtaggacagtgcagaca	57291-57311; 57579-57599	309	
12+13	ttgccacccagtagtatcca	tgcagggtgtggacaaagaa	62617-61636; 63113-63132	516	
14	ggcaacaagagcgaaact	gaggctactatgtgcttgtg	63941-63958; 64406-64425	478	
15	tttgccaagagacctacgg	tttctgtcttgcccactccc	65482-65500; 65951-65970	483	55
16	ttttctggaggagggcg	agggcatctctgtcttttgc	66514-66530; 66920-66939	426	
17+18	ccggttaccatcactgtccc	tgactacacacaattccccgg	68139-8158; 68808-68828	690	
19+20	tactgccactgtgacacgtg	gaaatcccatcggtgcatgc	69311-69330; 69740-69759	449	58
21	tctagatgctaccctgtgtgg	tcagtcatgcacagggttgg	73475-73495; 73754-73773	299	

[a] According to NCBI reference sequence NG_007097.2

PCR conditions consisted in an initial denaturation at 98ºC for 30 s, followed by 35 cycles of 98ºC for 10 s, an annealing temperature at either 55ºC or 58ºC for 10 s, and extension at 72ºC for 20 s; a final extension step for 5 minutes was performed. Each PCR reaction was carried out in a total volume of 25 µL containing nuclease-free water, PCR

buffer (5x), 3.0 mM $MgCl_2$, 3% dimethyl sulfoxide, 200 μM of deoxynucleotide triphosphate, 0.5 μM of each primer, 1.0 unit of Phusion® High-Fidelity DNA Polymerase (New England Biolabs), and approximately 200 ng of genomic DNA. To assure PCR products were amplified with the right dimension, an aliquot of each sample was run on a 2% agarose gel.

Total RNA isolated from lymphocytes was reverse transcribed using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Each RT-PCR reaction was prepared using a Reaction Mix containing reaction buffer, deoxynucleotide triphosphates, single-stranded 18-mer oligonucleotides and random hexamer primers, Maxima Enzyme Mix, with both reverse transcriptase (RT) and ribonuclease inhibitor, RiboLock, and finally, 1 μg of RNA, forming a total volume of 20 μL. The conditions for cDNA synthesis consisted in a first step at 25ºC for 10 minutes to allow primers to bind to the transcripts, followed by a second step at 50ºC for 15 minutes so that the RT can synthesize the cDNA strands, and a final step at 85ºC for 5 minutes to denature and degrade the remaining RNA. The total cDNA yield was quantified using NanoDrop ND-1000 spectrophotometer and stored at -20ºC until further use. PCR amplification was performed from *SERCA2* cDNA previously synthesized, using six sets of primers, also specifically designed with Primer3 program, which span the whole sequence of *SERCA2b* that is transcribed (Table 3).

For cDNA amplification, each PCR was performed according to PCR protocol for Phusion® High-Fidelity DNA Polymerase, as previously described, with annealing temperatures of 60 or 61ºC, a final magnesium chloride concentration of 1.5 mM or 3.0 mM, and a total volume of 25 μL.

Table 3 | PCR primers and related parameters for amplification of *SERCA2* from cDNA.

Exon	Forward Primer	Reverse Primer	Coordinates[a] (Forward; Reverse)	Product size (bp)	Annealing Temperature (ºC)
1-6	aagaggaggaggggagac	ttcacctgtgagaattgactgg	417-435; 1071-1092	676	61
6-8	tggtgacaaagttcctgctg	tgcagactgacatctggtttg	1008-1027; 1616-1636	629	60
8-13	gctcttggaactcgcagaat	accccactctcgaatgacag	1501-1520; 2180-2199	699	
13-15	acccacattcgagttggaag	gccacaatggtggagaagtt	2116-2135; 2755-2774	659	
15-18	gtgaacgatgctcctgctct	aaagtccgggttgtcctctt	2653-2672; 3170-3189	537	
17-20	ctgctgcatggtggttcat	ttcagcagaaatagacagttgctt	3092-3110; 3731-3754	663	

[a] According to NCBI reference sequence NM_170665.4

3.5. Sequence analysis

PCR and RT-PCR products, generated from genomic and coding sequences of *ATP2A2* respectively, were purified either from solution or TBE agarose gel slices, using Isolate II PCR and Gel Kit (Bioline). The clean-up protocol was based on a silica-membrane method which encompasses DNA-membrane fixation, membrane washing and DNA elution. PCR forward and/or reverse primers were added to the purified DNA fragments from each individual sample and submitted to GATC Biotech for bi-directional Sanger sequencing. This method uses terminator fluorescent dideoxynucleotides that allow the detection of each distinct nucleotide by laser and automatic storage of data in a computer. As a result, a series of fluorescence intensity peaks correspondent to each nucleotide can be observed in a chromatogram [56]. All chromatograms corresponding to PCR and RT-PCR fragments were carefully analyzed and the pathogenic mutation was found.

3.6 Quantitative PCR

The expression of *ATP2A2* gene was quantified by real-time PCR using the Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific) and the CFX96 Touch™ Real-Time System (Bio-Rad). Two sets of primers were designed to amplify both mutant and wild-type transcripts (GGTGACAAGTTCCTGCTGA forward and AGTGTGCTTGATGAC AGAGACA reverse), or the wild-type transcripts only (TGTGCTCTTTGTAATGACTCTGC forward and AGAAAGACCCTTCAATTCGGTATCA reverse).

The set of primers designed strictly for wild-type transcripts allows the amplification of exon 10, which is missing in mutant mRNA while the set of primers for both wild-type and mutant anneals in the region of exons 6 and 7. The objective of this experiment was to assess relative levels of mutant and wild-type transcripts and look for a link between these expressions and the variable phenotype between the affected relatives. *ATP2A2* mRNA expression was normalized by co-amplification of the housekeeping gene *ACTB*, coding for beta actin, which worked as an internal control. Primers used for evaluation of *ACTB* expression were a generous gift from Professor Graça Soveral

(CTGGGACGACATGGAGAAAATCTG forward and AGCCTGGATAGCAACGTACATG reverse).

3.7 Western blotting

Lymphocytes isolated from 3 mL of whole blood were homogenized with RIPA lysis and extraction buffer (Pierce, Thermo Fisher Scientific) supplemented with Halt™ Protease and Phosphatase Inhibitor Cocktail (100x) (Thermo Fisher Scientific), followed by sonication. Lymphocyte homogenates were centrifuged for 10 minutes at 8000 rpm and the soluble fraction was collected for Western blot analysis to determine the expression level of the mutant protein in comparison with the wild-type SERCA2. Different procedures for protein extraction were performed in order to find the best strategy to assess SERCA2 expression levels.

A quantity of 25 μg of total protein from each individual (evaluated by the Bicinchoninic Acid method) was mixed with Laemli loading buffer. Two sets of samples were studied as heated and unheated samples. The heated samples were denaturated at 95ºC for 5 minutes. All samples were briefly centrifuged and soluble fraction of cell lysates were resolved in 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (GE Healthcare Life Sciences, Amersham Hybond) at 300 mA for 100 minutes at 4ºC. Blots were blocked with a solution containing 5% bovine serum albumin (BSA), TBS (1x), and 0.1% Tween® 20 for one hour at room temperature. Membranes were immediately incubated with rabbit polyclonal anti-SERCA2 (dilution 1:10000; #4388 Cell Signaling) overnight at 4ºC, with gentle shaking. Membranes were probed with anti-rabbit IgG horseradish peroxidase-linked secondary antibody (dilution 1:2000; #7074, Cell Signaling Technologies, Inc.), followed by enhanced chemiluminescent blotting using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). Only after SERCA2 revelation the blots were incubated with primary antibody anti-β-actin (dilution 1:4000; Sigma-Aldrich), followed by secondary antibody incubation and revelation.

3.8 Genotype-Phenotype correlation

Since the family under study presents phenotypic variability among the affected individuals, it would be interesting to look for a genotype-phenotype correlation. Accordingly, all chromatograms from genomic DNA PCR products were analyzed closely in search for additional polymorphisms or insertions/deletions within exonic and intronic regions amplified by PCR, Additionally, *ATP2A2* and SERCA2 expression levels would also be taken into account for the search of genotype-phenotype correlation.

4. Results and Discussion

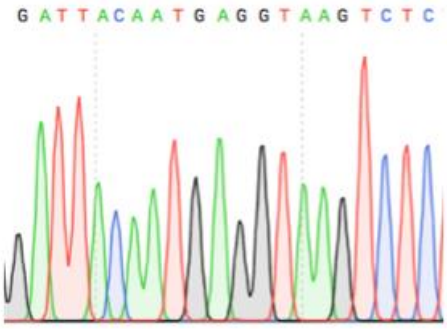
4.1 Identification of the pathogenic mutation

Bi-directional sequence analysis of the genomic DNA revealed a transversion in the first nucleotide of intron 10 (c.1287+1G>T or IVS10+1G>T), which corresponds to the G of the wild-type splice donor site dinucleotide (Figure 8A1). Therefore, the pathogenic mutation is considered a splice-site mutation, as proven by 2% gel electrophoresis (Figure 8A2) and bi-directional sequencing of RT-PCR products spanning exons 8 to 13, which confirmed that this variant affects the splicing process, leading to a shorter transcript where the skipping of full exon 10 is observed (Figure 8B). This mutant transcript contains a deletion of 103 base pairs (c.1185_1287del), originating a frameshift which putatively introduces a downstream premature stop codon (p.V395=fs*19). It was also observed that all four patients carry the same mutation in heterozigosity, whereas the unaffected individual carries the wild-type sequence in both alleles. For this reason, the unaffected individual was used as a negative control for the following experiments.

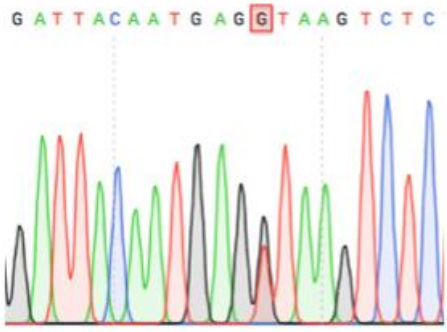
To understand what the splice site mutation does, it is important to know that splicing requires remarkable precision, which is granted by highly conserved intronic and exonic sequences and regulatory elements that are recognized by the spliceosome, a complex of small ribonucleoproteins (U1, U2, U5, U6/U4) and associated proteins [73]. Highly conserved intronic and/or exonic sequences include (1) the splice donor site GU and acceptor AG dinucleotides, recognized by the major spliceosome, at the 5’ and 3’ ends of the pre-mRNA, respectively; (2) the 5’ donor and 3’ acceptor splice site consensus sequences immediately upstream and downstream the GU and AG dinucleotides; and (3) the branch site, approximately 18 to 40 bp upstream the 3’ acceptor splice site [73]. Other relevant exonic and intronic sequences play a regulatory role either by promoting (splicing enhancer sequences) or inhibiting (splicing silencer sequences) the splicing process [73]. For instance, exon splicing enhancers are thought to compensate for the presence of splice sites with lower consensus values since it has been revealed that these sequences are in higher abundance in exons with “weak” splice acceptor sites [74].

1) Exon 10 + intronic boarders

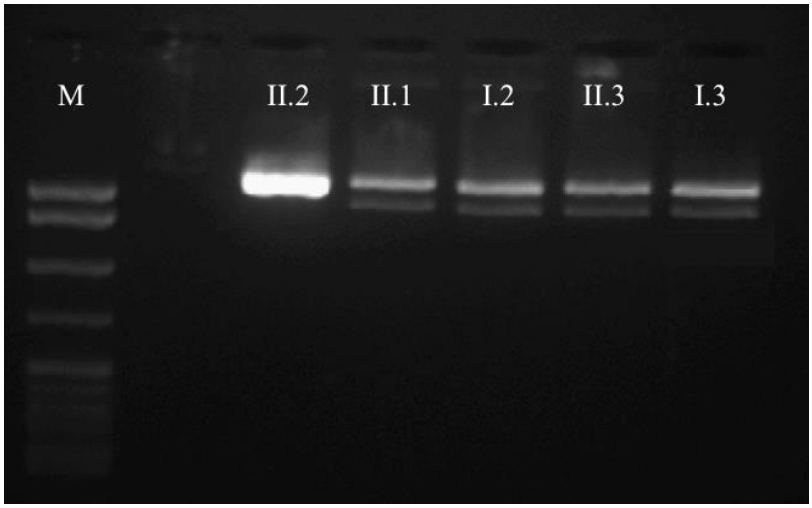
Wild-type allele



Mutant allele
c.1287+1G>T in heterozygosity



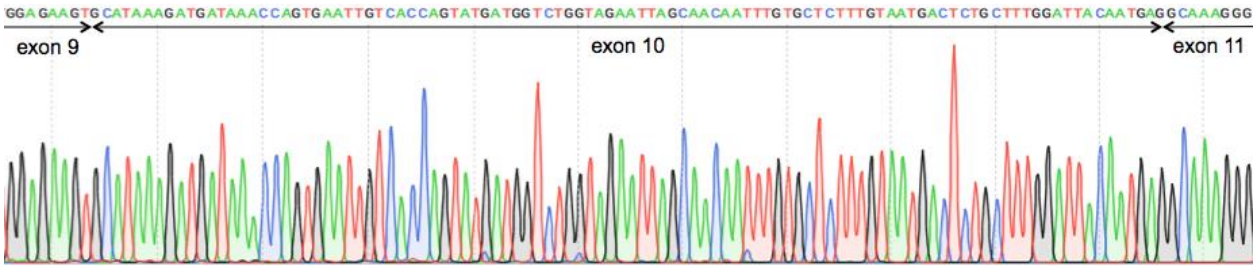
2) RT-PCR products (exon 8 – exon 13)



M- Molecular marker (pBR322 x MspI, New England Biolabs)

Figure 8A | Identification and expression of *ATP2A2* pathogenic mutation. (1) Sequence analysis of the genomic sequence of *ATP2A2* gene, corresponding to exon 10. The transversion IVS10+1G>T resulted in a splice-site mutation in intron 10. (2) RT-PCR products from every patient revealed that the splice site mutation yielded one wild-type allele transcript and a shorter transcript, while RT-PCR product from the unaffected member revealed two wild-type allele transcripts.

1) Fragment of cDNA sequence originating from the wild-type allele



2) Fragment of cDNA sequence originating from the mutant allele

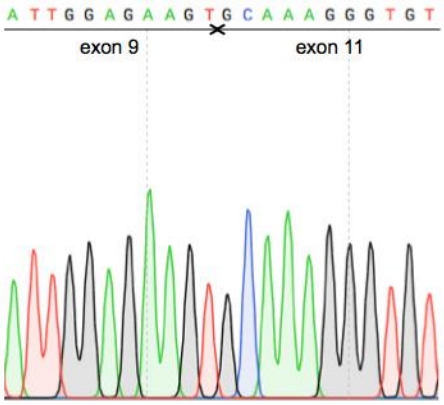


Figure 8B | Sequence analysis of the coding sequence of *ATP2A2* gene. Chromatograms correspondent to the wild-type sequence (1) and to the mutant sequence (2) which confirm the skipping of the entire region of exon 10 from every smaller transcript.

Mutations in these highly conserved sequences can either result in two consequences: the most frequent one, exon skipping, when an exon is no longer recognized as such, or cryptic splice site use, when the resulting mature RNA lacks a portion of an exon or contains an additional sequence from an intron. In the reported case, the reason why c.1287+1G>T led to exon skipping rather than cryptic splice site may rely on potential spliceosome's functional

response influencing factors, such as (1) local sequence context of the affected exon-intron junction and vicinities. For example, if the branch site of intron 10 exhibits a higher homology than the branch site sequence of intron 9; or if alternative splice sites with sufficient homology to the consensus sequence located in the vicinity are absent [73, 75]. (2) RNA secondary structure, since single stranded RNA might form base-pairing interactions and confer an unknown secondary structure to the mutant pre-transcript molecule *in vivo*, which might affect the access to the spliceosome [75]. And finally, (3) open reading frame (ORF) conservation since, in literature, it is postulated that exon skipping represents an evolutionary strategy towards better efficiency and stability as a spliceosome's functional response to splice site mutations since it preserves the homology in the ORF of the mRNA molecule [75].

4.2 *ATP2A2* expression

Cells have evolved quality-control mechanisms to deal with their naturally limited precision of cellular and molecular processes. In eukaryotes, abnormal mRNA molecules with premature translation termination codons (PTCs) can sometimes be substrate to the surveillance pathway of Nonsense-Mediated Decay (NMD). According to the 50-55 bp rule, a PTC located less than 50-55 bp upstream the 3'-most exon-exon junction is not recognized by the NMD surveillance system and the respective mRNA is translated, generating a truncated protein [76, 77]. If the PTC is located upstream the 50-55 bp region, the abnormal mRNA can either be immediately degraded by NMD or have its translation repressed in case it escapes NMD [78]. Nevertheless, there have been reported exceptions to this rule [78, 79].

In the studied case, the PTC derived from the skipping of exon 10 is located 74 bp upstream the 3'-most exon-exon junction (exon 11 – exon 12) (Figure 9). It is therefore expected that the abnormal transcript suffers nonsense codon recognition after splicing, which would lead to reduction of mRNA abundance by NMD.

Wild-type *ATP2A2* mRNA

1681 aa ggt gat act tgt tcc ctt aat gag ttt acc ata act gga tca act tat gca cct att g

E9 | E10
1741 ga gaa gt | g cat aaa gat gat aaa cca gtg aat tgt cac cag tat gat ggt ctg gta gaa t

E10 | E11
1801 ta gca aca att tgt gct ctt tgt aat gac tct gct ttg gat tac aat gag | gca aag ggt g

1861 tg tat gaa aaa gtt gga gaa gct aca gag act gct ctc act tgc cta gta gag aag atg a

1921 at gta ttt gat acc gaa ttg aag ggt ctt tct aaa ata gaa cgt gca aat gcc tgc aac t

E11 | E12
1981 ca | g tca tta aac agc tga tga aaa agg aat

Mutant *ATP2A2* mRNA

1681 aa ggt gat act tgt tcc ctt aat gag ttt acc ata act gga tca act tat gca cct att g

E9 | E11
1741 ga gaa gt | g caa agg gtg tgt atg aaa aag ttg gag aag cta cag aga ctg ctc tca ctt g

1801 cc tag tag aga aga tga atg tat ttg ata ccg aat tga agg gtc ttt cta aaa tag aac g

E11 | E12
1861 tg caa atg cct gca act ca | g tca tta aac agc tga tga aaa agg aat

PTC 50-55 bp region Exon | Exon

Figure 9 | Representation of fully spliced wild-type *ATP2A2* product versus mutant *ATP2A2* product. Exon-exon boundaries are indicated in green. The exon skipping-resulting frameshift starts in exon 11 (mutant *ATP2A2* mRNA), which leads to a putative premature translation termination codon (PTC), identified in red, located upstream the 50-55 bp region, identified in blue.

The findings illustrated in figure 8A2 suggest that a small amount of the mutant transcript (596 bp fragment) escapes NMD. Additionally, the band corresponding to the wild-type transcript (699 bp fragment) suggests an expression 50% inferior than that of the unaffected individual, who possesses an RT-PCR fragment equivalent to two copies of the wild-type *ATP2A2* gene. In order to confirm this prediction, expression levels of both wild-type- and mutant-allele-encoded transcripts were assessed by quantitative PCR. Results revealed a reduction of approximately 50-55% of wild-type plus mutant *ATP2A2* expression when compared to the healthy subject, whose *ATP2A2* gene expression was considered 100 % (Figure 10A). Next, the levels of wild-type gene expression alone were examined, allowing to conclude that the portion of wild-type mRNA from patients in figure 10A corresponds to approximately 36%, which leaves the mutant mRNA expression around residual levels of 18% (Figure 10B). The levels of wild-type and mutant transcripts detected by qPCR are in agreement with figure 8A2, which suggests that out of all the mRNA molecules produced during transcription of mutant *ATP2A2* gene, 18% fail to be recognized by NMD and could potentially lead to the generation of residual amounts of abnormal SERCA2.

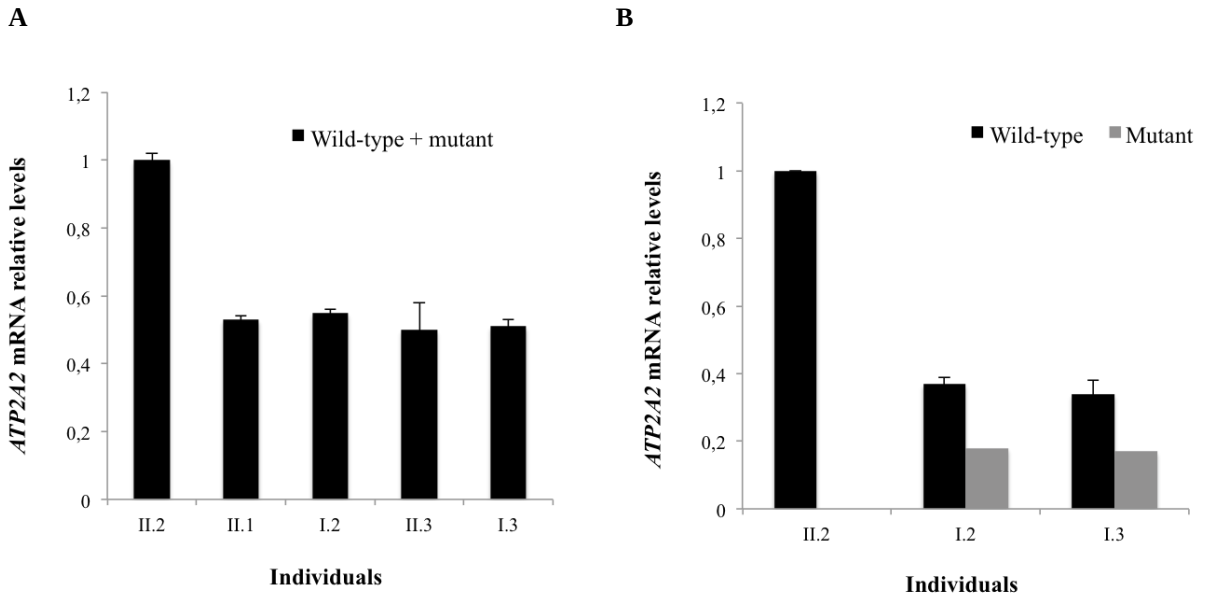


Figure 10 | All patients showed *ATP2A2* mRNA levels lower than 50% and residual levels of mutant mRNA. Quantitative PCR analysis was performed to quantify **(A)** expression levels of both wild-type and mutant transcripts and **(B)** wild-type transcripts alone. The data are presented as the mean ± SD of three independent experiments.

4.3 SERCA2 expression

If 18% of the mutant mRNA ought to generate an abnormal protein, this would be truncated in the C-terminal end, containing 413 amino acids rather than the expected 1042 (Figures 11A and 11B). The peptide chain would be normal until Val395, which is still preserved, but the remaining amino acids, from residues 396 to 413, would be different from the wild-type ones.

Wild-type SERCA2

1 MENAHTKTVE EVLGHFGVNE STGLSLEQVK KLKERWGSNE LPAEEGKTLL
51 ELVIEQFEDL LVRILLLAAC ISFVLAWFEE GEETITAFVE PFVILLILVA
101 NAIVGVWQER NAENAIEALK EYEPEMGKVY RQDRKSVQRI KAKDIVPGDI
151 VEIAVGDKVP ADIRLTSIKS TTLRVDQSIL TGESVSVIKH TDPVPDPRAV
201 NQDKKNMLFS GTNIAAGKAM GVVVATGVNT EIGKIRDEMV ATEQERTPLQ
251 QKLDEFGEQL SKVISLICIA VWIINIGHFN DPVHGGSWIR GAIYYFKIAV
301 ALAVAAIPEG LPAVITTCLA LGTRRMAKKN AIVRSLPSVE TLGCTSVICS
351 DKTGTLTTNQ MSVCRMFILD RVEGDTCSLN EFTITGSTYA PIGE**VHKDDK**
401 **PVNCHQYDGL VELATICALC NDSALDYNE**A KGVYEKVGEA TETALTCLVE
451 KMNVFDTELK GLSKIERANA CNSVIKQLMK KEFTLEFSRD RKSMSVYCTP
501 NKPSRTSMSK MFVKGAPEGV IDRCTHIRVG STKVPMTSGV KQKIMSVIRE
551 WGSGSDTLRC LALATHDNPL RREEMHLEDS ANFIKYETNL TFVGCVGMLD
601 PPRIEVASSV KLCRQAGIRV IMITGDNKGT AVAICRRIGI FGQDEDVTSK
651 AFTGREFDEL NPSAQRDACL NARCFARVEP SHKSKIVEFL QSFDEITAMT
701 GDGVNDAPAL KKAEIGIAMG SGTAVAKTAS EMVLADDNFS TIVAAVEEGR
751 AIYNNMKQFI RYLISSNVGE VVCIFLTAAL GFPEALIPVQ LLWVNLVTDG
801 LPATALGFNP PDLDIMNKPP RNPKEPLISG WLFFRYLAIG CYVGAATVGA
851 AAWWFIAADG GPRVSFYQLS HFLQCKEDNP DFEGVDCAIF ESPYPMTMAL
901 SVLVTIEMCN ALNSLSENQS LLRMPPWENI WLVGSICLSM SLHFLILYVE

Exon 10 (Val395-Glu429)

cDNA length (bp)	Protein length (aa)	Protein weight (kDa)
3126	1042	114,62

Figure 11A | Wild-type SERCA2b polypeptide sequence. Amino acids encoded by exon 10 is represented in violet. Wild-type SERCA2b parameters such as cDNA length, protein length, and protein weight are indicated below the polypeptide sequence.

Mutant SERCA2

1 MENAHTKTVE EVLGHFGVNE STGLSLEQVK KLKERWGSNE LPAEEGKTLL
51 ELVIEQFEDL LVRILLLAAC ISFVLAWFEE GEETITAFVE PFVILLILVA
101 NAIVGVWQER NAENAIEALK EYEPEMGKVY RQDRKSVQRI KAKDIVPGDI
151 VEIAVGDKVP ADIRLTSIKS TTLRVDQSIL TGESVSVIKH TDPVPDPRAV
201 NQDKKNMLFS GTNIAAGKAM GVVVATGVNT EIGKIRDEMV ATEQERTPLQ
251 QKLDEFGEQL SKVISLICIA VWIINIGHFN DPVHGGSWIR GAIYYFKIAV
301 ALAVAAIPEG LPAVITTCLA LGTRRMAKKN AIVRSLPSVE TLGCTSVICS
351 DKTGTLTTNQ MSVCR**MFILD RVEGDTCSLN EFTITGSTYA PIGEVQRVCM**
401 **KKLEKLQRLL SLA**

Exon 9 (Met366-Val395) **Exon 11 (Gln396-Ala413)**

cDNA length (bp)	Protein length (aa)	Protein weight (kDa)
1239	413	45,43

Figure 11B | Putative C-terminus truncated mutant SERCA2 polypeptide sequence. Amino acids encoded by exon 9 and exon 11 are represented in green and blue colors, respectively. Mutant SERCA2b parameters such as cDNA length, protein length, and protein weight are indicated below the polypeptide sequence.

All mutant SERCA2 splicing variants would lose several highly conserved amino acids and motifs since it would no longer have most of P-domain, and completely lack the nucleotide-binding and hinge domains, the 5th stalk domain, the transmembrane domains 5 to 10 or 11, where the calcium binding sites are located (TM5, 6, 8) and the 2b tail in case of the ubiquitous SERCA2b, which contributes to its high affinity for Ca^{2+} (Figure 12).

Therefore, when assessing expression levels of SERCA2 protein, it might be expected to observe a band around 115 kDa corresponding to the wild-type pump, and another band of 45 kDa corresponding to the mutant pump. When comparing heat-denaturated samples with unheated samples, it was found that heating prevented the detection of SERCA2 monomers in the blot, while enhancing the amount of oligomer (data not shown), as previously described [60]. Results show that wild-type SERCA2 proteins, when normalized to β-actin, present expression levels between 40 and 50% in DD patients when compared to the healthy individual (Figure 13), while no sign of mutant SERCA2 was detected (Figure 14).

There are three main explanations for the absence of mutant SERCA2 detection: (1) the amount of mutant SERCA2 derived from 18% of abnormal mRNA might be insufficient to generate detectable levels of protein; (2) the mutant protein is too immature to be stable and is immediately degraded by proteasomes; or (3) the remaining 18% PTC-containing mRNA molecules are not degraded by the NMD system but their translation is repressed by Nonsense Mediated Translational Repression (NMTR) mechanism, hence the synthesis of truncated protein is inhibited [78].

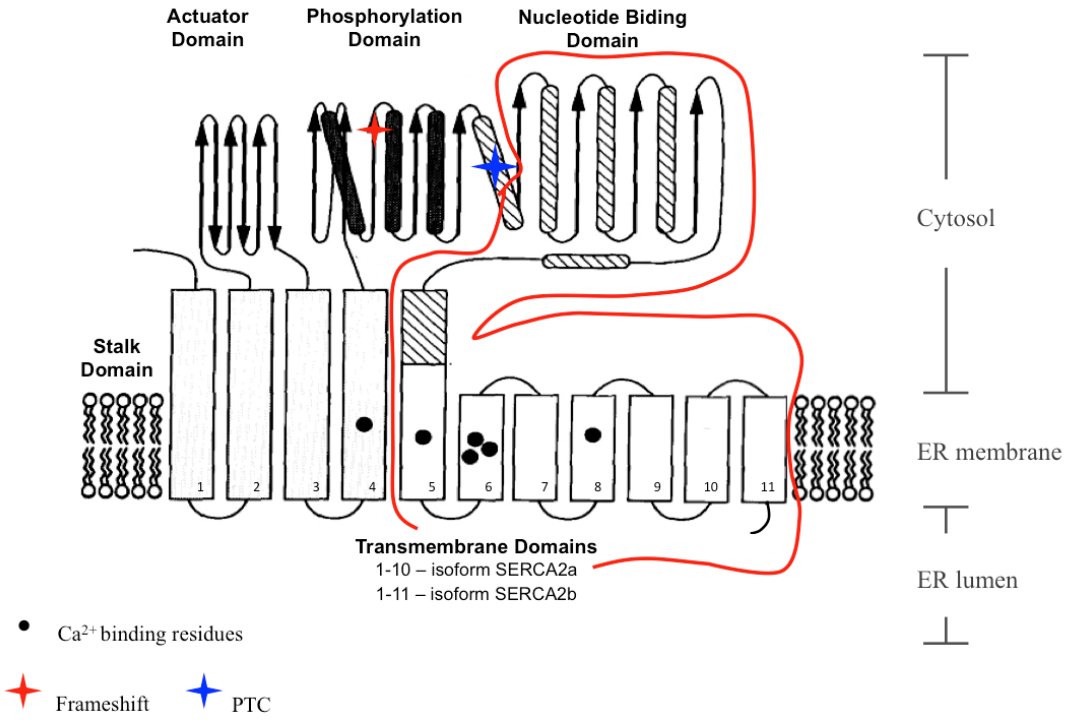
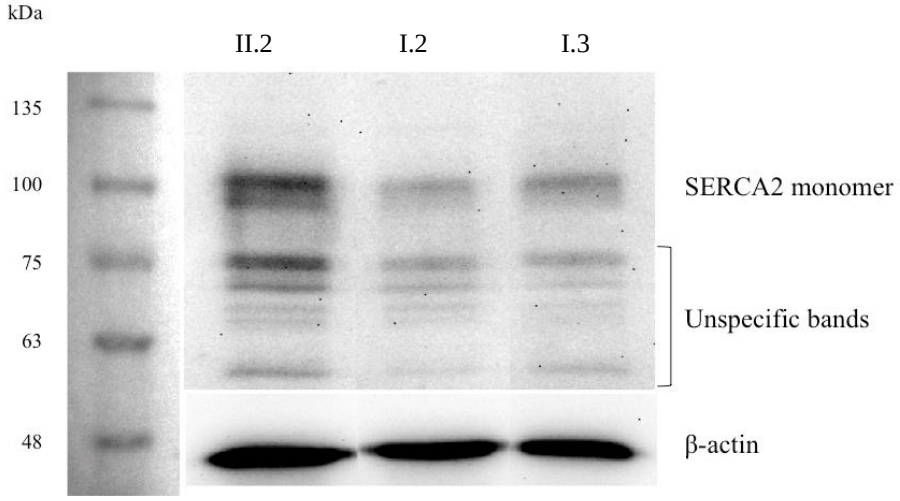


Figure 12 | Representation of SERCA2 domains and consequences of *ATP2A2* mutation. Frameshift would start in exon 11 which encodes for P-domain and N-domain. However, since the frameshift leads to a premature STOP codon, the putative SERCA2 mutant would lack all the following domains (within red line).

A strategy to examine whether the mutant SERCA2 is synthesized and potentially interacts with wild-type SERCA2 to form dysfunctional dimers, as previously reported [61], would be to co-immunoprecipitate both proteins from cultured keratinocytes obtained from the patients. This method would require the use of two primary antibodies: one antibody specifically designed for mutant SERCA2, which could hybridize with an epitope within the

frameshift-encoded region of the polypeptide; and an antibody specific for wild-type SERCA2, that could hybridize with an epitope within the region encoded by exon 10, which is missing in the mutant protein.

A



B

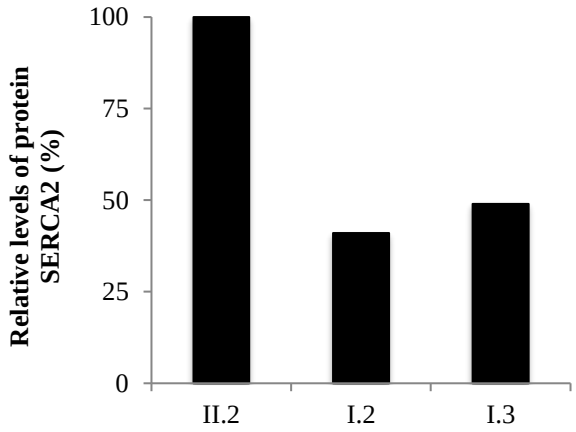


Figure 13 | Wild-type SERCA2 expression was severely reduced in patients when compared with the unaffected individual. (A) Naturally occurring SERCA2 proteins were severely reduced in affected individuals. Lymphocytes were lysed with 0.1% SDS and the resulting supernatants were resolved by 7.5% SDS-PAGE loaded with reduced, unheated samples. Anti-SERCA2 antibody was used for the western blots. After being washed, the same membrane was blotted with anti-beta-actin as the loading control. The marker used (left) was NZYColour Protein Marker II (Nzytech). (B) Percentage of wild-type SERCA2 Expression. Band intensity of SERCA2 was normalized with beta-actin.

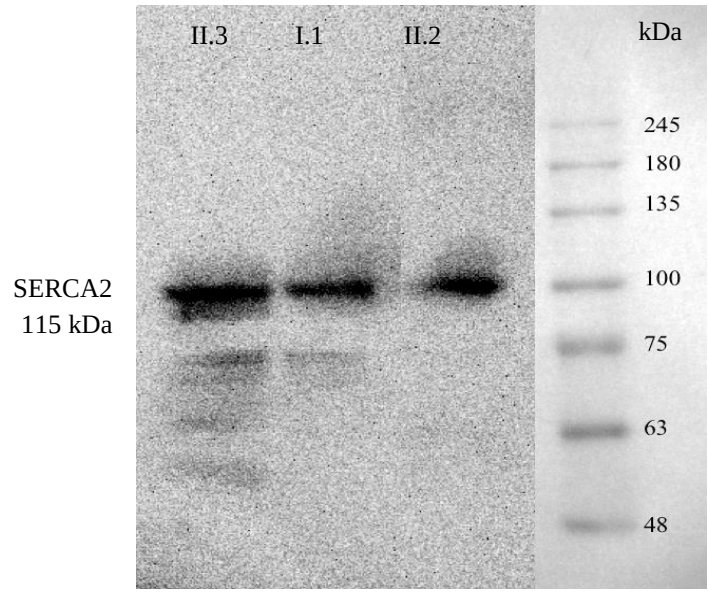


Figure 14 | Absence of truncated protein in DD patients. Putative mutant SERCA2 (45 kDa) could not be detected by western blot. Lymphocytes were lysed with 0.1% SDS and the resulting supernatants were resolved by 7.5% SDS-PAGE loaded with reduced, unheated samples. Blots were incubated with anti-SERCA2 antibody only, without posterior anti-beta-actin incubation so that the band corresponding to beta-actin would not mask the presence of a band of 45 kDa, corresponding to the putative truncated SERCA2. The marker used (right) was NZYColour Protein Marker II (Nzytech).

To assess whether SERCA2 mutants are degraded by proteasomes, skin biopsies would be necessary so cells could be treated with a proteasome inhibitor such as MG132, followed by western blot analysis of SERCA2 [60]. In case the amount of mutant SERCA2 fails to be detected when treated with the proteasome inhibitor, proteasome degradation hypothesis could be refuted.

On the other hand, a way to examine whether PTC-containing *SERCA2* mRNA is not detected due to translational repression rather than instability of mRNA could be by polysome analysis, which would require the establishment of DD cell lines from the patients [80, 81]. This technique separates translated mRNAs on a sucrose gradient according to the number of bound ribosomes. Polysomal fractions can be analysed by RT-PCR and its relative levels in each fraction would reveal the translational status of the mutant *SERCA2* mRNA [81]. Studies point that translational repression might depend on a putative *cis*-element residing in 3'-UTR, such as DNA transposons [80, 82]. Shen *et al.* revealed that miniature inverted-repeat transposable elements (MITEs) in the 3'-UTRs function as important regulators of translation,

therefore these could be potential *cis*-element candidates [82]. Nonetheless, the role of retrotransposons in regulating the metabolism of mRNA should be further investigated in human cells.

In general, the results herein presented strongly suggest that haploinsufficiency is the mechanism underlying DD pathology in these patients, since a single normal copy of *ATP2A2* gene seems insufficient to maintain a normal phenotype.

4.4 Genotype-phenotype correlations

4.4.1 Neuropsychiatric features

Calcium homeostasis depends on the rapid redistribution of Ca^{2+} ions into subcellular organelles, such as the ER, Golgi apparatus, and mitochondria, which serve as Ca^{2+} stores. The ER-Ca^{2+} stores rely on IP_3Rs, RyRs and SERCA pumps. Despite the restricted tissue expression of SERCA2 isoforms a and c, SERCA2b is ubiquitously expressed in every cell, including neurons. A disruption of SERCA2-Ca^{2+} transport may consequently disrupt vital cellular/neuronal activities given calcium‘s role as second messenger in various intracellular cascades. Disruption of normal Ca^{2+} signaling has been associated with neuropsychiatric symptoms, such as depression and epilepsy, whose incidence is higher in DD population than that in general population [83].

In this case report, depressive moods and single suicide attempt have been reported in patient II.1 while patient II.3 has been diagnosed with epilepsy. The remaining patients do not show clinical history of neuropsychiatric symptoms. Moreover, additional nucleotide alterations were not detected within the amplified and sequenced PCR products spanning exons and exon-intron boundaries. This suggests that neuropsychiatric phenotypes are not a direct consequence of *ATP2A2* mutations since the associated neuropsychiatric phenotypes in patients II.1 and II.3 are distinct and not segregated between the four relatives carrying the same *ATP2A2* mutation. In agreement, Ruiz-Perez et al. support the absence of association between neuropsychiatric features and mutation class in DD patients [84]. According to these findings, the effects of *ATP2A2* mutation in the brain may be a secondary consequence, however inconsistent. Nevertheless, *ATP2A2* contains several introns with extensive size that

were not amplified; hence the absence/presence of nucleotide alterations or even large insertions or deletions in these regions were not examined.

According to previous studies, only lesional keratinocytes present cadherin disruption although ER Ca^{2+} levels are reduced in both lesional and non lesional keratinocytes. Besides their fundamental role in epitelial adhesion, cadherins are very important in neuronal functions too, such as synaptic specificity, synapse function, and plasticity [45, 83]. Literature points to the possibility that cadherins might be key regulators of neuron-neuron connectivity since it has been demonstrated their circuit specific expression throughout the brain [40]. However, the question "How?" remains to be answered.

Since every cell is depleted of Ca^{2+} in the ER, it is reasonable to suggest that ER Ca^{2+} depletion is not enough to cause mislocalization and altered trafficing of cadherins. Rather, altered Ca^{2+}-induced cadherin expression, accumulation of somatic mutations in cadherin genes, and environmental factors might be underlying causes of this event. In fact, alterations in distinct cadherin genes have been associated with various neurological diseases [40].

4.4.2 Renal impairment

Besides neuropsychiatric disorders, previous reports have noted that DD patients may also present renal complications such as, polycystic kidney disease, renal agenesis, and horseshoe kidney [54, 55]. This is the first case reporting a patient (II.1) with DD in association with membranous nephropathy (MN), also known as membranous glomerulonephritis. Herein, it cannot be concluded whether this association with DD is by chance, therefore further studies on the incidence of renal impairment in DD population should be encouraged.

It is well established that ER stress is one of the major causes of kidney diseases [85]. As similarly reported in keratinocytes, ER stress-inducing agents, tunicamycin and thapsigargin, induce ER stress and autophagy in glomerular epithelial cells [60, 86]. In MN, immunoglobulin G and the complement form immune deposits in the subepithelial/intramembranous region of the glomeruli, which cause podocyte damage. Furthermore, as *ATP2A2* mutations notably lead to desmosomal disruption in keratinocytes due to intracellular calcium imbalance, these could also contribute, in combination with environmental factors or medication, to the alteration of podocytes' surface molecules by an

increase in ER stress, which may cause an immune response with antibody binding, complement activation, and cell damage [86, 87].

Idiopathic MN is more common in adults of advancing age while secondary MN is caused by infections (hepatitis B and C, HIV, syphilis), tumors or medicines [88]. However, since patient II.1 is negative for the infections above-mentioned, it is important to record this case in order to alert physicians for a possible link between DD and renal impairment.

4.4.3 Corneal susceptibility to viral infection by Herpes Simplex Virus (HSV)

Ocular involvement in DD remains relatively uncommon when compared with neuropsychiatric symptoms. Reported ocular features include dry eye syndrome with and without Sjögren's syndrome, cystic lesions in the upper eyelid, recurrent herpes simplex keratitis, and corneal manifestations such as corneal ulceration, perforation, and peripheral epithelial nebular opacities, associated with irregular surface of central corneal epithelium [56, 57, 89, 90].

The keratotic debris of DD patients is vulnerable to localized or widespread recurrent colonization by bacteria, fungi, or virus. Impaired desmosomes of the corneal epithelium may reduce corneal protection and potentiate microbiological colonization of the skin and ocular surface [90]. Moreover, the impairment of adhesion molecules at the cell boarders, the loss of subsets of dendritic cells in the skin, and defect in local immune response might influence the susceptibility for viral and bacterial infections in DD patients, as could be the case of patient I.2 [91, 92].

Adhesion molecules, such as e-cadherin, are known to facilitate lymphocyte homing, migration and distribution into epithelial tissue. Since lesional epidermis exhibits disrupted calcium signaling and altered e-cadherin traffic and localization, there is a huge chance that homotypic (e-cadherin-e-cadherin) and heterotypic ($\alpha^{E}\beta_{7}$ integrin-e-cadherin) adhesive interactions between intraepithelial lymphocytes and epithelial cells are impaired in DD pathology [93, 94]. With disrupted cadherins, leukocytes cannot be properly guided to the site of viral or bacterial infection. Therefore, it can be concluded that the increased risk of ocular

complications in DD may include herpes virus infection, as it occurs in patient I.2, who was already submitted to two cornea transplants.

4.4.4 Otological involvement

In these patients, hearing also seems to be affected by DD hallmarks, acantholysis and dyskeratosis, which lead to brownish-crusted papules in the outer ear, including the auditory canal, as previously described [95]. DD pathology in the ear has shown more severe consequences in patient II.1, whose hearing capacity from left ear is mostly loss and in need of surgical intervention.

Overall, no genotype-phenotype correlations have been found to support intrafamilial phenotypic variability. In conclusion, this phenomenon may rely on the influence of other nearby genes, pleiotropy of SERCA2, environmental factors, accumulation of somatic mutations, or pure chance. Moreover, since DD is a dominant condition, the phenotype of heterozygous individuals becomes more prone to external influences [96].

5. Conclusion

Mutations responsible for DD are scattered over the entire *ATP2A2* gene, hence it is not surprising that not every *ATP2A2* mutation lead to DD through one single main mechanism: haploinsufficiency or dominant-negative effect. In this family, splice-site mutation c.1287+1G>T appears to cause DD phenotype through haploinsufficiency, i.e. *in vivo*, 40-50% of SERCA2 normal function is not enough to maintain a normal Ca^{2+} signaling in the skin, which is the organ that is mostly exposed to outside influence. The pathogenic variant detected in this family has been previously found in a Japanese female, but described at DNA level only [97]. In Portugal, the clinical aspect of DD has been described in a 28-year-old male patient [98].

As the first molecular study of DD pathology in Portuguese patients, as well as the first molecular investigation of c.1287+1G>T at the transcriptional and translational levels, it adds knowledge to the worldwide mutational spectrum of the disease and, importantly, it contributes to genetic counseling since it allowed to discard a possible carrier status of the unaffected member of the family. This study also alerts physicians to the wide range of clinical manifestations in DD patients, including neuropsychiatric features, hearing loss, and corneal viral infections by HSV and glomerular nephritis which is, to our best knowledge, here firstly reported in association with DD.

Future perspectives would involve sequencing the promoter region of *ATP2A2* gene in order to examine whether other variants could explain the wild-type *SERCA2* mRNA expression levels below 50% that were observed in the patients. Furthermore, it would be relevant to sequence 3'UTR of *SERCA2* mRNA given that this region is thought to hold additional *cis*-NMD regulatory elements.

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